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## Successes of Modelling Parkinson Disease in *Drosophila*

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### 1. Introduction

Over one hundred years of innovative experimentation with the “common fruit fly” or *Drosophila melanogaster* has placed this remarkable organism at the forefront of contemporary biological research. Whether we consider the implications of modern genetic technologies and comprehensive genomic research, or we are interested in leading-edge aspects of molecular and cellular biology or complex developmental biology systems, or our studies range from the pin-point accuracy of proteomics to the large-scale questions of population biology, research with fruit flies has made very significant contributions to our understanding of the basic and complex functions of life. Although research into a wide range of biological questions has benefited greatly from experimentation with *Drosophila*, it never fails to surprise how often an approach that uses this model organism is undervalued or ignored. Any experimental system can be and should be criticized; however the opportunity to explore the biological basis of disease should never be missed. One shining example of this point is our understanding of Parkinson disease which has expanded through and continues to benefit from basic research into the biology of *Drosophila*, a model organism whose genome has been so thoroughly understood as to make it indispensable for medical research. These recent advances provide significant support for the use of *Drosophila melanogaster* models in the study of the biological basis of many human diseases and disorders.

Parkinson disease is the most common movement disorder and the second most common neurodegenerative disorder. Most apparent to even the most casual of observers is the fact that patients with Parkinson disease present with symptoms that are related to locomotion and motor control. These symptoms include resting tremor, slowness of movement, rigidity and postural instability. As the common underlying source that gives rise to these movement difficulties, Parkinson disease is most often associated with and distinguished by the degeneration of neurons, especially the dopamine-producing, or dopaminergic, neurons in the *substantia nigra* of the midbrain and the subsequent loss of dopamine (Dauer & Przedborski, 2003). Associated with these degenerating neurons is the appearance in many cases of the disease of large aggregates of proteins that are often referred to as the Lewy bodies. Often ignored are the additional non-motor symptoms, where non-dopaminergic neurons including olfactory and brain stem neurons, can frequently deteriorate before the dopaminergic neurons show signs of loss (Braak et al., 2003). Additional Parkinson disease symptoms are common and comprise a number of behavioural symptoms that include

dementia, depression, anxiety and difficulties with sleep, plus non-behavioural symptoms such as the development of muscular and skeletal anomalies and skin lesions (Simuni & Sethi, 2008). Given the importance and the complexity of this disease, the application of a multifaceted, interdisciplinary approach to understanding the biological basis of Parkinson disease, including the modelling of the disease in well-known genetically versatile organisms, cannot be stressed enough.

The inherited or familial forms of Parkinson disease are, for the most part, indistinguishable in nature and severity from the sporadic forms of the disease (Hardy et al., 2009). As of only a few years ago, Parkinson disease was believed to be completely sporadic in nature, yet the identification of the inherited forms of the disease along with subsequent characterization of the causative genetic defects has revolutionized this area of research. It is now known that the familial cases comprise approximately 10 to 15% of the cases and arise from mutations in several identified genes with new loci associated with Parkinson disease being routinely discovered. Of the loci identified early in this process, mutations in the *alpha-synuclein* gene (designated as both *PARK1/PARK4*) (Polymeropoulos et al., 1997; Singleton et al., 2003) and *Leucine-rich repeat kinase 2* or *LRRK2* (identified as *PARK8*) (Paisan-Ruiz et al., 2004; Zimprich et al., 2004) cause autosomal dominant or “gain-of-function” forms of the disease. Mutations in the *parkin* gene (designated as *PARK2*) (Kitada et al., 1998), *PTEN-induced kinase 1* or *Pink1* (*PARK6*) (Valente et al., 2004), and *Dj-1* (*PARK7*) (Bonifati et al., 2003) are associated with autosomal recessive or “loss-of-function” forms of Parkinson disease. With the identification of the underlying genetic contributions to, at the very least, a sizable proportion of the incidents of Parkinson disease, it has become possible to apply the principles of disease modelling in genetically tractable animal systems to the study of this disease.

To demonstrate the great utility of the application of research into *Drosophila melanogaster* in the modelling of Parkinson disease, I will describe some of the most exciting recent advances in this field. To begin, a brief description of the wealth of genetic and transgenic approaches that are most commonly used to model aspects of human disease in *Drosophila* will be provided as an introduction to the organism. The first model of Parkinson disease, one based upon the toxic expression of the human *alpha-synuclein* gene, the first gene identified as a genetic cause of Parkinson disease, will be discussed. This model offered the opportunities to study a wide range of biological contributions to Parkinson disease including aspects of protein structural stability, oxidative stresses and other disease genes. Investigation of *Lrrk/LRRK2* in flies has revealed roles in dopamine distribution, protein synthesis and cell death in another model of a dominant form of Parkinson disease. Then, the processes of modelling Parkinson disease through examination of the loss-of-function of the *Drosophila* homologues of the *parkin* and *Pink1* genes, both responsible for recessive forms of Parkinson disease will be detailed. Of a very significant nature, this area of research has led to the fundamental understanding the activity of these gene products at the mitochondria. Furthermore, recent studies have led to the proposal of a mechanism that outlines the normal role of *parkin* and *Pink1* in mitochondrial dynamics. It is very likely that the loss of this mechanism leads to failure of the cell's ability to clear damaged mitochondria and ultimately results in the degeneration of these cells and, subsequently, the disease state. Finally, continued study of *Drosophila* models of Parkinson disease is clearly well positioned to contribute a great deal to the future of research into the basis of this disease.

## 2. The *Drosophila* approach to model human disease

*Drosophila melanogaster* has been extensively studied and a wealth of genetic, genomic, cellular and developmental knowledge and reagents are readily available. *Drosophila* are inexpensive to propagate and can produce a large number of genetically homogenous progeny. Flies display surprisingly intricate behaviours and have complicated brain and nervous systems. For many purposes, the fly provides a well-characterized system that is relatively easy to manipulate but complex enough to be relevant to the development of human disease models.

Often, once a gene has been implicated in a given disease, a bioinformatic search of genomic sequences can readily identify a potential homologue or orthologue from among the genes that comprise the well-characterized genome of *Drosophila melanogaster* as well as the genomes of a number of other *Drosophila* species for comparison. Application of reverse genetics can lead the production of both loss-of-function and gain-of-function phenotypes that may recapitulate symptoms of a given disease. Loss-of-function can be achieved either through the creation of mutations that lower or abolish gene activity or through the directed expression of an interfering RNAi transgene. A gain of function phenotype can be generated by the directed expression of a gene to where there is normally low or no expression or by inducing elevated levels of expression far above the norm. As a very well studied system, the *Drosophila*'s pre-existing loss-of-function mutations, as well as transposon-bearing lines that can be employed to direct the expression, are available through stock centres and individual research laboratories from around the world. Due to the genomics projects, it is easy to access cDNA and genomic clones along with various vectors for generation of a variety of transgenic animals.

### 2.1 *Drosophila* genetics: genes, mutants and transgenics

In most cases, genes that have been identified as playing a role in human disease have counterparts in the genome of *Drosophila melanogaster*. Through the analysis of pre-existing mutants, the application of genetic screens to generate novel loss-of-function mutants and the implementation of "interfering RNA" or RNAi technology to reduce or eliminate gene activity can mimic the effect of recessively inherited diseases. For dominantly inherited disease, some pre-existing dominantly inherited alleles may exist in the stock centre collections. However, hypermorphic gain-of-function phenotypes can be generated by the directed overexpression of a transgene introduced into the fly genome through germ-line transformation. In addition, neomorphic and antimorphic gain-of-function phenotypes may be produced by *in vitro* construction and transformation of *Drosophila* transgenes that replicate molecular defects that have been determined to cause disease in people. Alternatively, a transgene that can express a variant form of a human gene can be fashioned and transformed. This approach has been applied to study the function and the potential contribution of genes to a number of diseases, including Parkinson disease. This approach can be instrumental in providing insight into the function of an identified gene involved in a human disease when the function is difficult to determine.

To produce the gain-of-function or the RNAi loss-of-function phenotypes described above, the Gal4/UAS system has been widely employed for the ectopic expression of specific genes in *Drosophila* (Brand & Perrimon 1993). For the most part, a gene of interest is cloned within a P element transposon-bearing plasmid containing several copies of the DNA-binding target of the yeast transcription factor GAL4 designated as the 'Upstream Activating

Sequence' (UAS) along with a selectable marker to readily track the presence of the transgene. As there is no *Drosophila* transcription factor that acts through binding of this sequence, in the absence of GAL4 these fusion transgenes are mostly inactive. Once established, individuals bearing a responsive transgene can be mated to specific lines that express Gal4 in any one or combination of expression patterns. Many choices of expression are available including ubiquitous, pan-neural, dopaminergic neuron-specific, early or late in eye development, inducible by heat shock, and many more. When the Gal4 transgene and the UAS target gene are combined in the offspring of the controlled breeding experiments, the gene of interest is subject to control of expression with regard to level, timing and tissue specificity. Analyses of these progeny provide the opportunity to approach a wide range of fundamental biological investigations including the detailed modelling of human disease.

When dealing with living systems, and in particular when carrying out complex manipulation of a model organism, careful evaluation and consideration of the techniques employed are necessary. With this in mind, it is important to note that a very limited number of Gal4 transgenes had been demonstrated to lead to cell death: first in the neuron-rich compound eye (Kramer & Staveley, 2003) and later in the nervous system (Rezaval et al., 2007). This suggests that the use of the Gal4/UAS system requires some caution because there may be a compounding Gal4-effect in some experiments when interpreting experimental observations. As always, control experiments must be subjected to proper evaluation and scrutiny. Nevertheless, the Gal4/UAS ectopic gene expression system in *Drosophila* is an extremely powerful tool and is one of the reasons that modelling human disease in flies is such an attractive pursuit.

## 2.2 *Drosophila* dopaminergic neurons

For a model system to functionally approach a condition as complex as Parkinson disease, changes to specific tissues that result in recapitulation of phenotypes that resemble symptoms of the disease are key. The *Drosophila* adult brain has been characterized to contain clusters of dopaminergic neurons (Nassel & Elekes, 1992). The feeding of rotenone, the complex I inhibitor that initiates degeneration of dopaminergic neurons in mammals, can cause the loss of these clusters of dopaminergic neurons in flies (Coulom & Birman, 2004). This demonstrates that there is a susceptibility of dopamine-producing neurons to toxins that is conserved between mammals and flies. As described below, altering the expression of selected genes in these dopaminergic neurons has resulted in loss of the neurons coupled with an age-dependent loss of locomotor abilities. The basic similarities between the genetically manipulated *Drosophila* models and Parkinson disease patients, given that the loss of the dopamine-producing neurons and the subsequent change in behaviour occurs over time, suggests that significant aspects of the disease may be well modelled in flies.

## 3. The alpha-synuclein-dependent *Drosophila* model of Parkinson disease

Once the genetic basis of a familial form of Parkinson disease was identified (the *alpha-synuclein* gene or *PARK1* and, later, *PARK4* as well) and the molecular basis of the defect determined (as specific changes to the protein), exploring the function or dysfunction of this gene was greatly aided by study in the *Drosophila* system.



### 3.1 The alpha-synuclein gene (PARK1) models Parkinson disease in *Drosophila*

Although instances are rare, mutations that result in substitution of amino acid residues within the human alpha-synuclein protein, specifically A30P, A53T and E46K, produce a dominant autosomally inherited version of Parkinson disease (PARK1; Polymeropoulos et al. 1997; Kruger et al. 1998; Zarranz et al. 2004). An early onset familial version of Parkinson disease results when a duplication of the region bearing this gene produces an increase in gene copy number (PARK4; Singleton et al. 2003). Although abundant in Lewy bodies, the precise physiological function of alpha-synuclein is uncertain. Because two of the original designated PARK loci (PARK1 and PARK4) have mapped to the *alpha-synuclein* gene, the native role and consequences of dysfunction appear complex and deserve to undergo extensive evaluation.

It is of extreme importance to the modelling of Parkinson disease in *Drosophila* that the first and most exploited *Drosophila* model of Parkinson disease depends upon the Gal4/UAS system for the ectopic expression of various forms of the human alpha-synuclein gene (Feany & Bender, 2000). Expression of human wild-type and Parkinson disease-causing mutant forms of *alpha-synuclein* in *Drosophila* results in the loss of the dopamine-producing neurons. The loss of neurons is coupled with the loss of the ability to climb over time in adult flies. As well, the development of Lewy body-like cytoplasmic inclusions and degeneration of the retina occurs with the expression of A30P, A53T and wild-type versions of *alpha-synuclein* using different Gal4 drivers. The observed phenotypic end-points produced by the disease-associated forms of the alpha-synuclein protein appear to display some differences and may mirror aspects of the toxicity that lead to various sub-types of Parkinson disease. To be clear, bioinformatic analysis of the genome of *Drosophila melanogaster* has not identified genes that encode any member of the synuclein family of proteins, including alpha synuclein. However, the recapitulation of phenotypes in *Drosophila* caused by the toxicity of *alpha-synuclein* gene product that somewhat mimic the consequences of Parkinson disease certainly seems to validate such study in this model organism. As with Parkinson disease patients, *Drosophila* models of Parkinson disease involve multiple defects, the cellular basis that provides insight into the pathogenesis of Parkinson disease.

### 3.2 Controversial detection of neurodegeneration in the model

Briefly, visualization of the dopaminergic neurons in *Drosophila* brains or brain sections has been accomplished through two main methods: detection of the tyrosine hydroxylase enzyme via immunohistochemistry, or detection of a *green fluorescent protein* or GFP reporter gene placed under the control of the *tyrosine hydroxylase-Gal4* transcription factor. For the most part, the determination of the specific death of dopaminergic neurons over time in response to the expression of *alpha-synuclein* has been demonstrated reproducibly since development of the alpha-synuclein-induced *Drosophila* model of Parkinson disease (Feany & Bender, 2000; Auluck et al., 2002; Cooper et al., 2006; Wassef et al., 2007; Botella et al., 2008; Trinh et al., 2008). Not all studies could detect dopaminergic neurodegeneration using anti-tyrosine hydroxylase immunostaining (Pesah et al., 2005) or detection of transgenic GFP (Whitworth et al. 2006). Clearly differences in the approach or methodology used to measure loss of dopaminergic neurons can influence the sensitivity of the assay (Auluck et al., 2005). In addition, as a definite decrease in the strength of the nuclear GFP signal has been observed the dopaminergic neurons of ageing flies that express *alpha-synuclein*, (Botella

et al. 2008) a significant proportion of the differences could have easily been overlooked. In primary cultures of neurons cultivated from *Drosophila* that express *alpha-synuclein*, an *in vitro* model for Parkinson disease that shows great potential, the observed decrease in detection of GFP in these cultured neurons has been strongly associated with the early stages of apoptosis and signs of neurodegeneration (Park et al., 2007). Although this has been a contentious issue, the loss of immunological staining of the tyrosine hydroxylase enzyme, or detection of the tyrosine hydroxylase-responsive GFP reporter, seems to be very well correlated to neuronal dysfunction and degeneration.

This controversy highlights one great advantage of the study of Parkinson disease in an organism that presents complex phenotypes that reflect the consequences of the disease in humans. Of great importance, experiments where the loss of neurons is correlated to the loss of locomotor ability over time have provided sufficient comparison. For example, when oxidative stress is prevented, the loss of locomotor activity and the loss of dopaminergic neurons are diminished together in flies expressing *alpha-synuclein* (Pendleton et al., 2002, Yang et al., 2003, Wassef et al., 2007, Botella et al., 2008). As such, the loss of climbing ability seems to be a meaningful and modifiable phenotype that allows for the detection and validation of subtle influences.

### 3.3 Mechanisms to prevent alpha-synuclein-dependent toxicity

To address the possibility that chaperone activity may actively counteract protein toxicity, elevated expression of Heat Shock Protein 70 (HSP70) was demonstrated to toxicity of *alpha-synuclein* expression although aggregates were found (Auluck et al., 2002). Reduced chaperone activity contributed to increased loss of neurons resulting from expression of *alpha-synuclein*. Providing transgenic flies with geldanamycin, an inducer of chaperone activity, added to the food source contributed to survival of *alpha-synuclein*-expressing neurons (Auluck et al., 2005). The phosphorylation of *alpha-synuclein* at residue Serine residue-129, a modification often found in brains from Parkinson disease patients, apparently leads to toxicity (Fujiwara et al., 2002). In *Drosophila melanogaster*, study of the consequences of *in vivo* alteration of this site of phosphorylation suggests that this amino-acid residue is responsible for keeping the *alpha-synuclein* protein in a soluble form instead of in a state of aggregation (Chen & Feany, 2005). Prevention of phosphorylation at this site results in aggregation and reduced toxicity. This observation supports the hypothesis that the soluble form of the protein has a much greater potential for toxicity than does the non-phosphorylated form. Taken all together, this suggests that the process of aggregation acts as a protective cellular mechanism that works to neutralize the toxic forms of the *alpha-synuclein* protein.

While oxidative stress seems to contribute to Parkinson disease, a relationship to the mechanism behind *alpha-synuclein* toxicity is unclear. The degeneration of neuronal phenotypes induced by expression of mutant forms of *alpha-synuclein* is enhanced growth under conditions of hyperoxia while the elevated expression of the oxygen free radical scavenger superoxide dismutase suppresses the neuronal degeneration and the loss of locomotor activity over the lifespan of *alpha-synuclein*-expressing flies (Botella et al., 2008). To support the role of oxidative insult in the disease process, the *alpha-synuclein*-induced phenotypes are suppressed by other known antioxidants. These include the overexpression of methionine sulfoxide reductase and the supplementation of the *Drosophila* growth medium with S-methyl-L-cysteine (Wassef et al., 2007), and the induction of glutathione

synthesis or glutathione conjugation activity (Trinh et al., 2008). As the toxicity of alpha-synuclein is sensitive to oxidative stress, manipulation of antioxidants may make a significant contribution to modify these effects.

One of the great advantages of employing the *Drosophila* model is the ability to combine and evaluate various components identified to contribute to Parkinson disease in an animal model organism. The first example of combining gene products that are known to cause inherited forms of Parkinson disease was the demonstration that the overexpression of *parkin* can act to counteract the toxic effects of both wild type and mutant forms of alpha-synuclein to restore climbing ability and to prevent degeneration of the retina when co-expressed in the eye (Haywood & Staveley, 2004; 2006). Similarly, the directed expression of *Pink1*, an upstream activator of *parkin*, acts to restore locomotor abilities and prevent subtle developmental defects in the eye (Todd & Staveley, 2008). This approach demonstrates that the directed expression of some of the recessive Parkinson genes can act to balance defects caused by a dominantly inherited Parkinson disease gene.

#### 4. The LRRK2/Lrrk (PARK8) models of Parkinson disease in *Drosophila*

*Leucine-rich repeat kinase 2* or LRRK2 (first identified as PARK8) causes an autosomal dominant or “gain-of-function” form of Parkinson disease (Paisan-Ruiz et al., 2004; Zimprich et al., 2004). Dysfunction of LRRK2 has been mapped to several amino-acid substitutions in the protein and is very prominent among sporadic and inherited forms of Parkinson disease. The LRRK2 gene encodes a very large protein with a leucine-rich repeat (LRR) domain, a kinase domain, a RAS-like GTPase domain and WD-40 domain and is very similar to an orthologous gene LRRK1.

There is a single homologue, *Lrrk*, in *Drosophila melanogaster*. Perhaps due to difficulties in the detection of neurodegeneration, as discussed above, there has been some confusion with studies of *Lrrk*/LRRK2 in flies. In one case, *Lrrk* mutant flies display impaired locomotive activity and a reduction in the immunostaining of tyrosine hydroxylase in dopaminergic neurons (Lee et al., 2007). While the dopaminergic neurons display abnormal morphologies, the absolute number of the neurons appears to be unchanged although they may be degenerating slowly. Under other circumstances, *Lrrk* mutants seemed relatively normal which lead to the claim that *Lrrk* is not required for the survival of dopaminergic neurons (Wang et al., 2008). Directed expression of wild type of *Lrrk* did not lead to detectable degeneration of dopaminergic neurons (Lee et al., 2007). However, expression of mutant forms of both human (G2019S) and *Drosophila* (I2020T) did lead to documented loss of dopaminergic neurons (Imai et al., 2008; Liu et al., 2008). Nevertheless, the few studies of *Lrrk*/LRRK2 in flies have revealed a great deal that may influence our understanding of Parkinson disease.

The *Lrrk* mutants undergo lipid peroxidation and mutant flies containing the carboxy-terminal kinase domain truncated *Lrrk* transgene are sensitive to hydrogen peroxide (Wang et al., 2008). However, *Lrrk* mutants seem to be reasonably resistant to the oxidative stresses presented by paraquat and hydrogen peroxide (Imai et al., 2008). The relationship between *Lrrk*/LRRK2 and oxidative stress is unclear for now.

*Lrrk*/LRRK2 has been demonstrated to be involved in the negative regulation of normal levels of dopamine. The over expression of select mutants of *Lrrk*, but not wild-type *Lrrk*, causes a severe reduction in the dopamine levels of the brain (Imai et al., 2008). Treatment with L-DOPA causes improvement in movement not survival of dopaminergic neurons (Liu



et al., 2008). Conversely, dopamine content is highly elevated in *Lrrk* mutants, as changes in dopamine levels must not be due to survival neurons but due to either defects in metabolism or processing and handling.

The eukaryotic initiation factor 4E (eIF4E)-binding protein, a major controller of protein synthesis and as such a key regulator of responses to cellular stress is phosphorylated by the *Lrrk*/LRRK2 kinase (Imai et al., 2008). This strongly suggests that the survival of dopaminergic neurons is compromised by pathogenic forms of *Lrrk*/LRRK2 through the deregulation or mis-regulation of protein translation. The *Lrrk* and LRRK2 proteins have been shown to phosphorylate and activate the transcription factor foxo (Kanao et al., 2010). This suggests that downstream targets of foxo, such as *hid* in flies (and *Bim* in humans), act to activate the apoptotic machinery to cause the neurodegeneration in *Lrrk*/LRRK2 models of PD. These research findings demonstrate a very meaningful connection between the control of protein synthesis, activation of cell death programs and the development of Parkinson disease.

## 5. The parkin/Pink1-dependent Drosophila models of Parkinson disease

### 5.1 The consequences of parkin and Pink1 loss in Drosophila

The loss-of-function in the *parkin* gene (PARK2), which encodes a highly conserved ubiquitin E3 ligase, is responsible for a rare autosomal recessive subtype of Parkinson disease. The *Drosophila parkin* gene is highly expressed in the *Drosophila* central nervous system (Horowitz et al. 2001; Bae et al. 2003). In *parkin* mutants, the dopaminergic neurons degenerate (Greene et al., 2003; Cha et al., 2005; Whitworth et al., 2005; Wang et al., 2007). In flies that overexpress some mutant forms of *parkin*, dopaminergic neurons degeneration occurs (Sang et al., 2007). Although viable, *parkin* mutants present with a reduction in life-span, locomotor defects and extensive degeneration of muscle fibres, the latter is clearly associated with mitochondrial deterioration (Greene et al., 2005). The male *parkin* mutants are sterile due to failure of mitochondrial activities during spermatogenesis (Riparbelli and Callaini, 2007). Reduction of neuronal-specific staining (either GFP or TH) and/or cell death has been reported in these *parkin* mutants (Greene et al., 2003; Cha et al., 2005; Whitworth et al., 2005; Wang et al. 2007). The localization of this protein to the mitochondria (Darios et al., 2003) coupled with the consequences of *parkin* loss is a strong indication that it protects this organelle.

Mutations in the *PTEN-induced kinase 1* (*Pink1* or PARK6) gene are a common cause of autosomal recessive Parkinson disease (Valente et al., 2004). In flies, the *Pink1* gene, like *parkin*, is highly expressed in adult heads and testes (Park et al. 2006). The *Pink1* serine-threonine kinase, along with a kinase domain, contains a mitochondrial-targeting signal (Clark et al., 2006). A decrease in the levels of dopamine with age plus a somewhat limited loss of dopaminergic neurons was found in the *Pink1* mutants along with the presence of abnormal mitochondria in the surviving dopaminergic neurons. When the function of *Pink1* was inhibited through the directed expression of an RNAi transgene, loss of dopaminergic neurons as well as the age-dependent degeneration of ommatidia was observed (Wang et al. 2006; Yang et al. 2006). The potential for a functional association with the mitochondria and the similarity in the consequences of dysfunction clearly suggest a shared role for these two proteins.

### 5.2 The parkin and Pink1 proteins act in one pathway

Although not identical, the flies that have lost *Pink1* function share a number of defects found in the *parkin* mutants including shortened lifespan, apoptotic muscle degeneration,

male sterility, defects in mitochondrial morphology and disruption of locomotor abilities. These mitochondria are lost with age from the dopaminergic neurons of *Pink1* mutants. While, for the most part, double *Pink1-parkin* mutants show the same phenotypes as either of the single mutants, the overexpression of *parkin* is able to rescue the mitochondrial defects found in *Pink1*, whereas *Pink1* overexpression does not rescue the *parkin* phenotypes. The *parkin* and *Pink1* proteins have been reported to interact physically in at least some contexts (Kim et al., 2008; Xiong et al., 2009). This, at least in part, indicates that the *Pink1* and *parkin* proteins function in the same pathway with *Pink1* functioning upstream of *parkin* activity. This *Pink1/parkin* pathway is necessary for the integrity of dopaminergic neurons, because the loss of neurons due to loss of *Pink1* function could be rescued by additional expression of *parkin* (Clark et al., 2006; Park et al., 2006). The contribution of the study of the relationship between *parkin* and *Pink1* to our understanding of mitochondrial pathology in Parkinson disease highlights the utility of *Drosophila* to model Parkinson disease.

Similar to *parkin* and *Pink1*, mutations in the *Dj-1* gene cause autosomal recessive forms of Parkinson disease (Bonifati et al., 2003) and it has been suggested that they will become another potential component of this pathway. *Drosophila melanogaster* has two homologues of *Dj-1* and is viable when both are deleted or silenced by RNAi transgenes (Menzies et al., 2005; Meulener et al., 2005; 2006). The loss of *Dj-1* activities leads to increased sensitivity to oxidative stress when exposed to paraquat or rotenone. However, the overexpression of *Dj-1* does not rescue the *Pink1* mutant phenotypes (Yang et al., 2006). The possibility exists that *Dj-1* may act much further upstream or through a parallel mechanism. However, at this time the relationship is unclear and *Dj-1* may or may not influence the *Pink1/parkin* pathway.

### 5.3 The *parkin* and *Pink1* proteins co-operate at the mitochondria

Mitochondria undergo fission and fusion to change shape and share components (Chen & Chan, 2009). The fusion of mitochondria requires fusion of both the inner and outer mitochondrial membranes. The control of outer membrane fusion requires the activity of the protein mitofusin, and the inner membrane fusion requires the product of the *Optic atrophy-1* gene. Mitochondrial fission is promoted by the recruitment of dynamin-related protein 1 (*Drp1*) to the mitochondria, and this recruitment requires the activity of *Fis1*, a mitochondrial outer membrane protein. The regulation of this process is essential to the maintenance of a healthy cell.

To focus upon the mitochondria, in the dopaminergic neurons and the adult flight muscles of *parkin* mutants, the mitochondria are swollen with fragmented cristae (Greene et al., 2003; Pesah et al., 2004). A similar phenomenon is observed in flies that have lost the function of *Pink1* (Clark et al., 2006; Park et al., 2006; Yang et al., 2006). During *Drosophila* spermatogenesis, the spermatid's mitochondria aggregate and fuse to produce the nebenkern, a structure composed of two entangled strings of fused mitochondria. During subsequent elongation, the nebenkern disentangles to yield two fused structures that are maintained throughout the process of spermatogenesis. Whether due to reduced mitochondrial fission or excess fusion, in *parkin* and *Pink1* mutants only one mitochondrial fusion product has been detected (Deng et al., 2008). These mitochondrial defects, along with locomotion defects, flight muscle degeneration, cell death and diminishment of dopamine levels in heads, are suppressed both by the directed expression of the pro-fission genes *drp1* or *fis1* and by decreasing levels of the pro-fusion genes *mitofusin* or *opa1* (Deng et al., 2008; Park et al., 2009; Poole et al., 2008; Yang et al., 2008). In a *Pink1* mutant background,

a reduction in the gene copy number of *drp1*, as seen with mutant heterozygotes, causes lethality (Deng et al., 2008; Poole et al., 2008). This clearly suggests that *parkin* and *Pink1* must act to promote mitochondrial fission. It is extremely important to point out that the phenotypes that arise from the loss of *parkin* or *Pink1* are very different from the loss of *drp1* (Deng et al., 2008). This is a strong indication that the *Pink1*-*parkin* pathway acts to regulate mitochondrial fission machinery.

#### 5.4 The regulation of Mitophagy by *parkin* and *Pink1* proteins

The process of mitophagy is a recently described specialized mitochondrial-specific version of autophagy (Goldman et al., 2010). In this procedure, the mitochondria undergo engulfment by autophagosomes and are degraded. This essential mechanism is absolutely dependent upon the dynamics of the continual fission and fusion of the mitochondria that alter the size and shape of the organelle and allow the exchange of components. With the failure of mitophagy, the quality of cellular respiration is severely diminished as is illustrated through the accumulation of oxidized proteins. Through the regulation of the fission/fusion dynamics of the cellular mitochondria, the *Pink1* and *parkin* collaborate to contribute to this process.

First of all, although the *parkin* E3 ubiquitin ligase can target toxic proteins for proteasomal degradation, as the loss of *parkin* results in the accumulation of toxic proteins and overexpression can suppress toxicity of potential targets, other cellular processes can be regulated by ubiquitination by *parkin* (Geisler et al., 2009). With this in mind, a mechanism has been proposed that outlines the potential roles of the *parkin* and *Pink1* proteins in the regulation of mitochondrial dynamics, changes in which can lead to alterations in the process of mitophagy (Geisler et al., 2009; Narendra et al., 2009; Vives-Bauza et al., 2010; Ziviani et al., 2010). The recruitment of *parkin* from the cytoplasm to the mitochondria depends on the activity of the *Pink1* kinase. *Pink1* is localized to the outer mitochondrial membrane through a well-conserved mitochondrial targeting signal peptide located near the amino-terminus of the protein (Zhou et al., 2008). This arrangement leaves the carboxy-terminal kinase-containing remainder of the protein exposed to the cytoplasm. It is proposed that under normal conditions, the tethered *Pink1* protein is cleaved in a constitutive way to release the portion of *Pink1* that contains the kinase activity into the cytoplasm (Narendra et al., 2009; Vives-Bauza et al., 2010; Ziviani et al., 2010). As a result, under standard conditions, *Pink1* activity at the mitochondrial membrane is maintained at a steady but low level.

However, when stressed mitochondria undergo a critical amount of damage, the routine degradation of the *Pink1* kinase is discontinued (Narendra et al., 2009; Vives-Bauza et al., 2010; Ziviani et al., 2010). This leaves intact and active versions of *Pink1* to accumulate at the mitochondrial membrane in response to the termination of *Pink1* inactivation. The initiation and maintenance of the accumulation of *Pink1* may depend upon a signal generated when the mitochondria are not able to maintain membrane potential. Although the Rhomboid-7 protease is a candidate enzyme that can cleave *Pink1* (Whitworth et al., 2008), this protease may not be the one responsible for this activity in response to mitochondria signalling for destruction. In the first major step of this process, the result is the differential identification of damaged mitochondria through the build up of active *Pink1* activity bound and secured to the outer mitochondrial membrane. According to this mechanism, the next step in the regulation of mitophagy depends upon the recruitment of the *parkin* E3 ubiquitin ligase

though Pink1 activity to the outer mitochondrial membrane (Narendra et al., 2009; Vives-Bauza et al., 2010; Ziviani et al., 2010). Recruitment of parkin to the mitochondrial membrane depends upon the localization of the Pink1 kinase to the mitochondria. An alternative method of mitochondrial targeting of Pink1 also recruits parkin (Narendra et al., 2009). Finally, the presence of the parkin ligase at the mitochondria results in the ubiquitination and subsequent degradation of the fusion-promoting outer mitochondrial membrane protein mitofusin (Ziviani et al., 2010). The mitofusin protein has been shown to accumulate with the loss of *Pink1* and *parkin* gene functions. If this proposal holds true, the initiation of mitophagy may very well depend upon signaling through the ubiquitination of mitofusin. In turn, whether mitofusin is targeted for degradation or modified to a form that no longer contributes to the process, this situation leads to the prevention of the fusion of the outer mitochondrial membrane. Although there is no evidence that fission is directly influenced, the subsequent failure of damaged mitochondria to be isolated would likely result from their loss of the ability to undergo proper segregation.

In summary, when mitochondria accumulate sufficient damage, the Pink1 protein becomes stabilized at the mitochondria. This acts to recruit parkin to the damaged mitochondria that in turn causes degradation or modification of mitofusin to promote mitochondrial fission and mitophagy to remove these damaged mitochondria. In Pink1/parkin mediated Parkinson disease, the damaged mitochondria are not cleared as efficiently to result in cellular damage.

## 6. The future of drosophila models of Parkinson disease

*Drosophila* is proving to have great advantages in the genetic and cell biological study of Parkinson disease. As more genes are found to be associated with Parkinson disease, further applications of reverse genetics should lead to greater understanding of the disease. The *Drosophila* phenotypes offer many diverse clues that may benefit from greater scrutiny. As well as future screens that modify the more obvious phenotypes such as male sterility, muscle degeneration, abnormal wing positioning, locomotion defects as well as mitochondrial defects in multiple tissues in young adults, these can be examined/scored for suppression or enhancement without having to carry out aging studies that can span months. Studies of candidate genes that may modify the activities of genes that mediate familial Parkinson disease are rigorous, but allow for rapid and straightforward means to deduce mechanisms. Given that mitochondria defects accumulate during normal aging, identification of multiple means to activate mitophagy may have applications to many aspects of aging. *Drosophila* provides an indispensable and unique opportunity to contribute to the Parkinson disease field.

## 7. Conclusion

To summarize, the recent expansion of research interest in the well understood laboratory organism *Drosophila melanogaster* to provide highly informative models of Parkinson disease demonstrates some of the great advantages that this system has to offer. The first model of Parkinson disease, one based on the toxic expression of the human *alpha-synuclein* gene, has allowed a great deal of investigation into various biological factors, such as protein folding, oxidative stress and protein detoxification, that may contribute to the disease. Studies of



*Lrrk/LRRK2* have revealed roles for the management of dopamine handling, the control of protein synthesis and the initiation of programmed cell death in models of Parkinson disease. Further modelling of Parkinson disease through careful evaluation of *parkin* and *Pink1* loss-of-function in *Drosophila* has revealed that these two genes contribute to a pathway where the parkin E3 ubiquitin ligase is under the regulatory control of the Pink1 protein kinase. In flies, the relationship between *parkin* and *Pink1* has been extended from the neurons to other tissues including the mitochondrial-rich flight muscle and the male gametes. This has led to investigation of the products of these highly conserved genes in the processes that promote mitochondrial survival. The routine participation of *parkin* and *Pink1* in mitochondrial activities reveals that the loss of the fusion/fission dynamic can result in the failure of the cells to adequately deal with mitochondria that present a burden to the health of the cell. Future study of Parkinson disease in *Drosophila* models will continue to reveal much about the basis of this disease. As the past century has made clear, over and over again, investigations that apply our collective knowledge of the genetics and biology of *Drosophila melanogaster* to explore the fundamentals of disease, such as Parkinson disease, are very difficult to ignore.

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## **Mechanisms in Parkinson's Disease - Models and Treatments**

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Parkinson's disease (PD) results primarily from the death of dopaminergic neurons in the substantia nigra. Current PD medications treat symptoms; none halt or retard dopaminergic neuron degeneration. The main obstacle to developing neuroprotective therapies is a limited understanding of the key molecular mechanisms that provoke neurodegeneration. The discovery of PD genes has led to the hypothesis that misfolding of proteins and dysfunction of the ubiquitin-proteasome pathway are pivotal to PD pathogenesis. Previously implicated culprits in PD neurodegeneration, mitochondrial dysfunction, and oxidative stress may also act in part by causing the accumulation of misfolded proteins, in addition to producing other deleterious events in dopaminergic neurons. Neurotoxin-based models have been important in elucidating the molecular cascade of cell death in dopaminergic neurons. PD models based on the manipulation of PD genes should prove valuable in elucidating important aspects of the disease, such as selective vulnerability of substantia nigra dopaminergic neurons to the degenerative process.

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